

WE CLAIM:

1. Methylophilic recombinant yeast strain for producing human insulin precursor, the strain having a genome comprising a copy of a first DNA construction and a second DNA construction, wherein said constructions controlling the expression and secretion of human insulin precursor, said DNA constructions comprising at least one DNA sequence encoding a human insulin precursor and analogous thereof.

2. The yeast strain of claim 1, wherein the strain secreted a human insulin precursor of the formula:

B(1-30)-Y1-Y2-A(1-21), wherein Y1 is lysine or arginine; Y2 is lysine or arginine; B(1-30) is the B peptide of the human insulin; and A(1-21) is the A peptide of human insulin.

3. The yeast strain of claim 1, wherein the strain is a member selected from *Hansenula*, *Pichia*, *Candida*, and *Torulopsis*.

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5. The yeast strain of claim 1, wherein the first DNA construction comprises:

b1) an exporting signal sequence operable linked to

d1) a 3' termination sequence linked to

e1) a selectable gene linked to

f1) a second insertable DNA sequence; and

the second DNA construction comprises:

b2) an exporting signal sequence operable linked to

d2) a 3' termination sequence linked to

e2) a selectable gene distinct from the selectable gene of the first DNA construction.

6. The first DNA construction of claim 5, comprising at least one expression cassette for expressing the human insulin precursor, the cassette comprising:

- a) a 5' regulatory region operably linked to
- b) a DNA sequence encoding a signal sequence operable linked to
- c) a sequence encoding a human insulin precursor operable linked to
- d) a functional termination sequence.

7. The first DNA construction of claim 5, wherein the selectable gene is HIS4.

8. The first DNA construction of claim 5, wherein 5' and 3' ends of said DNA construction comprises sequences enough homologous with a target gene of the yeast to permit the replacement by a specific insertion of the DNA construction in the target gene, in the same relative orientation of the target gene in the yeast genome.

9. The first DNA construction of claim 5, wherein the 5' regulatory region is selected from the group consisting of the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *Pichia pastoris*

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dihydroxyacetone synthase (DAS) gene promoter, the *Pichia pastoris* p40 regulatory regions promoter, the *Pichia pastoris* catalase promoter, and the glyceraldehyde dehydrogenase GAP promoter.

10. The first DNA construction of claim 5, wherein the signal sequence is the MF α of *Sacharomyces cerevisiae*.

11. The first DNA construction of claim 5, wherein the functional termination sequence is the termination sequence derived from *Pichia pastoris* AOX1 gene.

12. The first DNA construction of claim 5, further comprising:

a) a first insertable DNA sequence corresponding to a 5' regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* operable linked to

c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) operable linked to

d) a 3' termination sequence of *Pichia pastoris* AOX1 gene operably linked to

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e) a *Pichia pastoris* HIS4 selection gene operably linked to

f) a second insertable DNA sequence corresponding to *Pichia pastoris* AOX1 gene termination sequence.

13. The first DNA construction of claim 5, wherein the sequence encoding the human insulin precursor is cloned in said construction following the protease site of the signal peptide, wherein all the secreted human insulin precursor contains, in its amino terminal end, the first amino acid of the human insulin precursor.

14. The first DNA construction of claims 5-13, wherein said DNA construction is incorporated into a vector selected from the group consisting of linear and circular vectors.

15. The second DNA construction of claim 5, comprising at least one expression cassette for expressing the human insulin precursor, the cassette comprising:

- a) a 5' regulatory region operably linked to
- b) a DNA sequence encoding a signal sequence operable linked to
- c) a sequence encoding a human insulin precursor operable linked to

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d) a functional termination sequence.

16. The second DNA construction of claim 5, further comprising a selectable gene distinct from the selectable gene of the first DNA construction, wherein said selectable gene is the gene encoding zeocine resistance and wherein said selectable gene permits to carry out a second selection event.

17. The second DNA construction of claim 5, wherein 5' end of said DNA construction comprises a single sequence enough homologous with a target gene of the yeast to permit the integration of the DNA construction in the target gene, in a single event.

18. The second DNA construction of claim 5, wherein the 5' regulatory region is selected from the group consisting of the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *Pichia pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *Pichia pastoris* p40 regulatory regions promoter, the *Pichia pastoris* catalase promoter, and the glyceraldehyde dehydrogenate GAP promoter.

19. The second DNA construction of claim 5, wherein the signal sequence is MF α *Sacharomyces cerevisiae*.

20. The second DNA construction of claim 5, wherein the functional termination sequence is the termination sequence derived from *Pichia pastoris* AOX1 gene.

21. The second DNA construction of claim 5, further comprising:

a) a first insertable DNA sequence corresponding to a 5' regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* operable linked to

c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) operable linked to

d) a 3' termination sequence of *Pichia pastoris* AOX1 gene operably linked to

e) the zeocine-resistant selection gene.

22. The second DNA construction of claim 5, wherein the sequence encoding the human insulin precursor is cloned in said construction following the protease site, wherein all the secreted human insulin precursor contains, in its

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23. The second DNA construction of claims 15-22, wherein said DNA construction is incorporated into a vector selected from the group consisting of linear and circular vectors.

i) transforming a yeast cell with a first DNA construction comprising:

b) the MF α signal sequence of *Sacharomyces cerevisiae* operable linked to

d) a 3' transcription termination sequence of *Pichia pastoris* AOX1 gene operably linked to

e) a *Pichia pastoris* HIS4 selection gene operably linked to

f) a second insertable DNA sequence corresponding to *Pichia pastoris* AOX1 gene termination sequence;

ii) selecting the yeast cells;

iii) isolating a yeast strain;

iv) re-transforming the yeast strain obtained in steps i)-iii) with a second DNA construction comprising:

a) a first insertable DNA sequence corresponding to a 5' regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* operably linked to

c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) operable linked to

d) a 3' termination sequence of *Pichia pastoris* AOX1 gene operably linked to

e) the zeocine-resistant selection gene;

v) selecting the re-transformed yeast strain; and

vi) isolating the selected and re-transformed yeast strain.

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